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13. ABSTRACT ***(Maximum 200 Words)***

Proteins that regulate progression through the G1 phase, including cyclin D1 and the cyclin-dependent kinase (cdk) inhibitors $p21^{cip1}$ and $p27^{kip1}$, appear to be particularly important in the pathogenesis of breast cancer. We suggested that aggressive breast cancer would involve both (i)overexpression of cyclin D1 and (ii) failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims were designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. We generated mouse embryo fibroblasts (MEFs) that stably and inducibly overexpress cyclin D1. In contrast to the data in NIH-3T3 cells, there was no compensatory upregulation of p21 in MEFs in response to cyclin D1 overexpression. An analysis of several established breast cancer cell lines has also failed to show a relationship between levels of cyclin D1 and the cdk inhibitors, supporting the MEF data. Expression of cyclin D1, p27, and p21 have been analyzed in samples of human lobular carcinoma by immunohistochemistry. It appears that, unlike ductal carcinoma, cyclin D1 is preferentially overexpressed in invasive components of lobular carcinoma. In addition, whereas there was no correlation beween cyclin D1 or p21 and proliferation markers, p27 levels demonstrated an inverse correlation with markers of proliferation in human lobular carcinoma.

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INTRODUCTION

An increasing body of evidence suggests that derangements in the cell cycle machinery contribute to uncontrolled cell growth and tumorigenesis. This is particularly true for those which control progression through the G1 phase, including cyclin D1 and the cyclin-dependent kinase (cdk) inhibitors $p21^{cip1}$ (p21) and $p27^{kip1}$ (p27). Cyclin D1 upregulation occurs as a result of gene amplification and/or mRNA overexpression in a substantial proportion of human breast cancers (1-5). Overexpression of cyclin D1 in transgenic mice leads to breast hyperplasia and multifocal carcinoma (6). Alternatively, in breast as well as other cancers, the cdk inhibitors appear to function as tumor suppressors. Thus, lower than normal protein levels correlate significantly with tumor aggressiveness, histologic grade, and decreased overall patient survival (7-11).

As positive and negative regulators of proliferation, respectively, the levels of cyclins and cdk inhibitors relative to each other determine whether progression through G1 phase proceeds (12-16). In addition, as key factors controlling G1 progression, all three are regulated by extracellular stimuli, including growth factors and cellular adhesion to the extracellular matrix (17,18). Such actions are responsible for the anchorage- and mitogen-dependence of G1 progression in normal cells. Conversely, anchorage- and mitogen-independence is the hallmark of tumorigenesis.

Whether the aberrant levels of cyclin D1, p21, and p27 result in altered cell cycle progression and subsequent proliferation remains to be established. Overexpression of cyclin D1 in cells can result in similar or even delayed cell cycle kinetics, compared to normal mammary epithelial cells (4, 19, 20). Moreover, the overexpression of cyclin D1 fails to induce anchorage-independent growth, the best in vitro correlate of tumorigenicity. Our preliminary data show that the consequences of cyclin D1 overexpression will be most apparent when examining anchorage-independent growth because that is the condition in which normal levels of cyclin D1 become rate-limiting. In addition, our data show that the overexpression of cyclin D1 in NIH-3T3 cells leads to a compensatory increase in the cdk inhibitor p21, and that this compensatory increase can counteract the expected cyclin D1 effect on anchorage-independent Rb phosphorylation and cell cycle progression. Thus, we proposed that the value of cyclin D1 overexpression as a diagnostic indicator for breast cancer is weakened by the compensatory upregulation of cdk inhibitors (CKIs) that can occur in breast cancer cells. We further suggest that aggressive breast cancer will involve both (i) overexpression of cyclin D1 and (ii) the failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims outlined below are designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. Aim 1 is to show that compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression in inducing anchorage-independent growth. Aim 2 is to determine if compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression on tumor formation. Aim 3 is to determine if the overexpression of cyclin D1 in breast cancer cell lines has a more pronounced growth effect if the cells have also lost their ability to upregulate CKIs. Aim 4 is to examine the relative expression of cyclin D1 and CKIs in a series of breast cancer biopsies.

BODY

Aim 1: Test the hypothesis that the compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression in inducing anchorage-independent growth.

Task 1. Complete characterization of adhesion-dependent phenotype of wild-type, p21, and p27 null mouse embryo fibroblasts (MEFs).

As demonstrated in previous reports, we have found that both p21-null and p27-null MEFs have partially lost the ability to down-regulate cyclin E-cdk2 activity when stimulated in suspension. However, they retain normal adhesion requirements for cyclin D1 expression, Rb phosphorylation, and cyclin A expression. These cell lines thus exhibit an adhesion-dependent phenotype despite the knock-out of one or the other cdk inhibitors.

Task 2. Obtain tetracycline-cyclin D1 transfectants in mouse embryo fibroblasts. Analyze the tetracycline-cyclin D1 cells (wild-type and knock-out) for their ability to undergo anchorage-independent growth. Compare the rates and extent of Rb phosphorylation, cyclins D1 and A expression, and p21 levels when the transfectants are cultured in the presence and absence of substratum.

We have previously indicated that MEFs were not efficient in their ability to form stable transfectants. However, we have been able to transfect early passage mouse embryo fibroblasts (MEFs) with a tetracycline-repressible cyclin D1 cDNA and isolate several clones that show tetracycline-regulated expression of cyclin D1. (In this system, tetracycline represses the expression of the ectopic cDNA and removal of tetracycline causes its induction.) The general goal is to determine if the induction of cyclin D1 leads to increased expression of p21 as we observed in NIH-3T3 cells.

We isolated three distinct clones that show tetracycline-regulated expression of cyclin D1, clones M13, M14 and M18 (Fig 1). The clones differ in the basal level of cyclin D1 expression (observed when cultured in the presence of tetracycline) and in the degree to which cyclin D1 expression is properly adhesion dependent (regulated correctly in clones M13 and M14, but apparently not so in clone M18). Importantly, all three clones show strong induction of cyclin D1 upon removal of tetracycline. p21 expression, as expected from our previous studies (17) is upregulated by culturing the cells in the absence of a substratum. However, p21 expression is *not* altered by the induction of cyclin D1 (Fig 1, compare p21 levels in the presence an absence of tetracycline). The inability of cyclin D1 overexpression to alter p21 is seen in both adherent and nonadherent cells from all three clones. Given these unexpected results, the proposed follow-up experiments to determine the impact of cyclin D1 overexpression on in vivo tumor formation of p21 null MEFs are no longer indicated or justified (aim 2 in the original application). We are presenting this data again because of its impact to obviate the justification for aim 2.

Aim 2: Determine if compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression on tumor formation.

As stated above, we observed no changes in the levels of p21 despite potently inducing cyclin D1 overexpression in MEFs. This lack of effect in MEFs is critical to approaching Aim 2 as this is the cell line where p21- and p27-null cells have been produced. The proposed experiments, to determine the consequences of compensatory p21 upregulation on tumor formation are therefore, no longer justified.

Aim 3: Determine if the overexpression of cyclin D1 in breast cancer cell lines has a more pronounced growth effect if the cells have also lost their ability to upregulate cyclin-dependent kinase inhibitors.

Task 1. Characterize a series of breast cancer cell lines which overexpress cyclin D1.

We have begun choosing a set of breast cancer cell lines to analyze for cyclin D1 and p21 expression. As part of this task, we needed a relatively "normal" breast epithelial cell line for comparison. We have chosen the MCF10A line because it is an immortal cell line that arose spontaneously from benign breast tissue without viral or chemical intervention (some of which can disrupt cell cycle regulation). In our hands the cells exhibit a requirement for mitogens and adhesion for proliferation. Asynchronous proliferating MCF10A cells exhibit low to moderate but easily detectable levels of cyclin D1. The levels of p21 and p27 are nearly undetectable (see below).

Most of the studies characterizing cell cycle events and their regulation by adhesion to the extracellular matrix have been performed in fibroblast cell lines. There is little information available regarding adhesion-dependent cell cycle progression in breast epithelial cells. In order to examine the effects of loss of adhesion, we have used cytochalasin D (CCD), a potent inhibitor of actin polymerization. Like suspension cultures, CCD treatment causes complete disruption of the cytoskeleton, unclusters integrins, induces a rounded cell shape, and has been shown to reproduce the same G1 effects as occur in suspended cells (21, 22). Treatment of quiescent MCF10A monolayers with CCD inhibits the mitogen-induced down-regulation of p21 and p27, supporting the adhesion-dependence of these events (Figure 2). Because it was unclear from previously reported experiments, additional studies have been performed to clarify the effects on cyclin D1 by CCD treatment. There is no apparent effect of CCD on cyclin D1, implying that cyclin D1 is not adhesion-dependent in breast epithelial cells (Figure 2). In contrast, indirect disruption of the cytoskeleton via inhibition of signaling molecules with toxin A, does inhibit cyclin D1. This result was unexpected because cyclin D1 is profoundly affected by CCD in numerous fibroblast cells.

We have subsequently begun comparisons with malignant breast cancer cell lines with regard to cyclin D1 and p21/p27 expression levels. We have analyzed the breast cancer cell lines MDA-MB-231, -468, and MCF-7 and made

comparisons with the nontransformed epithelial line, MCF10A. As compared to MCF10A cells, the malignant cells overexpress cyclin D1 during asynchronous growth (Figure 3 left). p27 and p21 levels are low in all the lines under these conditions. Furthermore, the tumorigenic lines MDA-MB-231 and –468 fail to upregulate p27 in response to serum and growth factor deprivation (Figure 3, right). Significantly, MDA-MB-468 cells also continue to express high levels of cyclin D1 even in the absence of mitogens (Figure 3, right).

Task 2. Using lines and results from Task 1, determine if the consequences of cyclin D1 overexpression is inversely correlated with expression levels of the p21-CKI family.

As seen from the expression levels in figure 3, there appears to be no clear relationship between the levels of cyclin D1 and the cdk inhibitors in asynchronously growing malignant breast cell lines. Likewise, under conditions of serum and growth factor deprivation, no clear correlation appears between these proteins. These results are consistent with the data obtained with ectopic overexpression of cyclin D1 in MEFs as shown in aim 1.

Aim 4: Examine the relative expression of cyclin D1 and cyclin dependent kinase inhibitors in a series of breast cancer biopsies.

Task 1. Develop and quantify the immunohistochemical procedures for the analysis of breast cancer biopsies. Confirm that the antibodies can detect protein expression in formalin fixed, paraffin-embedded tissue.

We determined the procedure for optimal staining of cell pellet sections for cyclin D1 and p27 in a previous report. We have since determined the optimal conditions low and high expression levels of p21 and have prepared cell pellets to analyze optimal conditions for p21 staining. Tissue sections undergo antigen retrieval by exposure to steam heat at 95° C for 20 minutes. Sections are incubated with the primary antibody for 3 hours at room temperature at a dilution of 1:1,000 for anti-p21. Subsequent incubation with secondary antibody and development with DAB chromogen is used following standard procedures. Counterstaining is with fast green.

Task 2: Obtain high- and low-expression controls for cyclin D1, p21, and p27 antibodies by preparing blocks from cell lines which over- or under-express the cognate protein.

We described the preparation and staining of the positive and negative controls for cyclin D1, p27, and p21 in previous reports.

Task 3. Identify samples of human breast cancer tissue specimens and prepare sections of these specimens in a blinded fashion for evaluation.

Dr. Mies has chosen 22 specimens of human lobular breast carcinoma tissue from the tissue bank at the University of Miami. Some of these contain components of in situ carcinoma in addition to the invasive components. Samples of lobular carcinoma were chosen because it is a subset of breast cancer (~10%) that has not been well-characterized with respect to cell cycle protein expression. In contrast, expression levels of cell cycle proteins have been well-described in samples of human ductal carcinoma (reviewed in 23, 24).

Task 4. Perform the immunohistochemical staining of the selected specimens using the characterized antibodies. Analyze and quantify the relative expression levels of cyclin D1, p21 and p27 in each biopsy.

Dr. Mies has performed the immunohistochemical staining for cyclin D1, p27, and p21 on the specimens chosen in Task 3. Dr. Mies has also stained the sections for MIB-1/Ki67, an S-phase marker. The immunohistochemical staining was scored as 1+ (1-33% cells positive), 2+ (34-66%), or 3+ (67-100%).

As shown in Table 1, 67% of invasive and 25% of in situ carcinoma samples overexpress cyclin D1. No such difference between invasive and in situ samples was observed with p21 or p27 staining. Cyclin D1 levels in the invasive component were equal to or greater than the corresponding in situ components in 100% of the cases (Table 2). Further analysis was performed using the proliferation marker MIB-1/Ki-67. As shown in Table 3, cyclin D1 levels did not correlate with high proliferation. In contrast, there was a strong correlation between high p27 levels and low proliferation. These results suggest that overexpression of cyclin D1 is not associated with hyperproliferation of human lobular carcinoma, whereas p27 expression appears to correlate inversely with proliferation. Unlike ductal carcinoma, cyclin D1 is preferentially overexpressed in invasive components of lobular carcinoma.

Task 5. Analyze the correlation between relative expression levels of cell cycle proteins and prognosis.

We have focused our immunohistochemical analysis on samples of lobular carcinoma, which forms a relatively uncommon subset of human breast cancer. Unlike the more extensively characterized ductal carcinoma, there are not large numbers of these specimens available for analysis. We have therefore made comparisons between levels of cyclin D1, p27, or p21 and proliferation markers as described above and shown in Table 3.

KEY RESEARCH ACCOMPLISHMENTS

-A tetracycline-regulated cyclin D1 construct has been successfully cloned into mouse embryo fibroblasts and is stable in cell culture. Cyclin D1 expression is normally regulated by adhesion in two of these clones and appropriately regulated by tetracycline in all.

-Utilizing these clones, extensive characterization of the effect of cyclin D1 on p21 expression has been performed. It appears that in mouse embryo fibroblasts, there is no compensatory increase in p21 levels despite significant overexpression of cyclin D1.

-We have characterized the conditions for regulation of cyclin D1, p27 and p21 in the nontransformed breast epithelial cell line MCF10A by growth factors and adhesion. Growth factors and adhesion appear to be required for p27/p21 down-regulation. In contrast, cyclin D1 expression appears not to be effected by adhesion or an intact cytoskeleton.

-We have examined several malignant breast cancer cell lines that overexpress cyclin D1 relative to nontransformed breast epithelial cells. There appears to be no relationship between high cyclin D1 expression and p27/p21 levels.

-Samples of human lobular carcinoma have been selected and analyzed immunohistochemically for cyclin D1, p27, and p21 expression levels. Expression levels in invasive and in situ components have been compared and the relationship between these proteins and proliferation markers have been analyzed.

REPORTABLE OUTCOMES

1. We have developed MEF cells that are stably transfected with a tetracyline-regulated cyclin D1 construct that are adhesion-dependent and appropriately responsive to tetracycline.

2. Some of the results of these studies have been presented as a poster at the DOD Breast Cancer Research Program Era of Hope Meeting in Atlanta in June 2000, "Cyclin D1, p27kip1, and p21cip1 in Human Lobular Carcinoma" (poster # D-2).

3. Welsh, C.F. and Mies, C. Expression of cyclin D1, p27kip1, and p21cip1 in human lobular carcinoma. Manuscript in preparation.

CONCLUSIONS ("so what section")

1. Mouse embryo fibroblasts that stably express a tetracycline-regulated cyclin D1 construct have been successfully established. This system was chosen because p21 and p27 knock-outs were generated in MEFs. Despite significant overexpression of cyclin D1 in these cells, there was no compensatory upregulation of p21 in MEFs. This is in contrast to the ability of NIH-3T3 cells to upregulate p21 in response to cyclin D1 overexpression. This result precludes the ability to analyze the effect of such interaction on tumor formation of these cells in nude mice.

2. The nontransformed breast epithelial cell line MCF10A exhibits an adhesion-dependent phenotype with respect to G1 cell cycle progression. The mitogen-induced down-regulation of p27/p21 appears to be dependent on cell adhesion, integrin clustering, and an intact cytoskeleton. In contrast cyclin D1 expression is independent of these factors. This result is in contrast to fibroblast cells and underscores the importance of characterizing fundamental characteristics of breast epithelial cells, which may differ from more well-characterized model systems. In addition, this result raises the possibility that overexpression of cyclin D1 may not contribute substantially to anchorage-independent growth in breast tumor cells.

3. Several human breast cancer cell lines were examined that overexpress cyclin D1 relative to MCF10A. In both asynchronously growing cells and during mitogen starvation, there was no apparent relationship between expression of cyclin D1 and 27/p21 levels. These data support the results from forced cyclin D1 expression in MEFs.

4. A series of human lobular carcinoma tissue specimens have been analyzed for cyclin D1, p27, p21, and MIB-1/Ki67 levels. We find that cyclin D1 is preferentially overexpressed in invasive versus in situ components, a feature which is distinct from ductal carcinoma. This may reflect specific differences in molecular changes that occur during the acquisition of the invasive phenotype between these two clinicopathologic entities.

5. p27 levels demonstrated an inverse correlation with markers of proliferation in human lobular carcinoma specimens, consistent with its role as a cyclin-dependent kinase inhibitor and its identification as a tumor suppressor. There was no correlation between cyclin D1 or p21 and markers of proliferation. These results are consistent with the idea that the prognostic significance associated with p27 levels in breast cancer is at least partially due to its effects on proliferation. These results also imply that the significance of cyclin D1 expression in breast cancer progression is not due primarily to its effects on proliferation.

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FIGURES

Figure 1. Control (untransfected) MEFs and MEFs stably expressing tetracycline-regulated cyclin D1 were serum-starved into G0, trypsinized, suspended in DMEM, 5% FCS and replated in monolayer (Mn) or suspension (Sp) in the presence (+) and absence (-) of tetracycline for 18 hr. Cells were collected and lysed. Equal amounts of protein from each cell lysate was fractionated on an SDS gel, transferred to nitrocelluolose and immunoblotted with antibodies specific for cyclin D1, p21 and cdk4 (protein loading control).

Figure 2. Quiescent MCF10a monolayers were treated with 2 ug/ml cytchalasin D (CD), toxin A (Tx, which indirectly disrupts the cytoskeleton through effects on signaling molecules) or untreated (-). Cells were stimulated with FCS and growth factors and harvested at the indicated times. Cyclin D1, p27, p21, and cdk4 (loading control) were determined by immunoblotting.

Figure 3. Breast epithelial (MCF10A) and breast cancer cell lines (MCF7, MDA-MB-468 and –231) were seeded subconfluently and harvested after 24 hrs as asynchronously growing monolayers (left). The indicated cell lines were also starved of serum and growth factors for 48 hrs prior to harvesting (right). Levels of cyclin D1, p27, p21 were analyzed by immunoblot. Actin was determined to be a more reliable indicator of loading than cdk4 when comparing different cell lines.

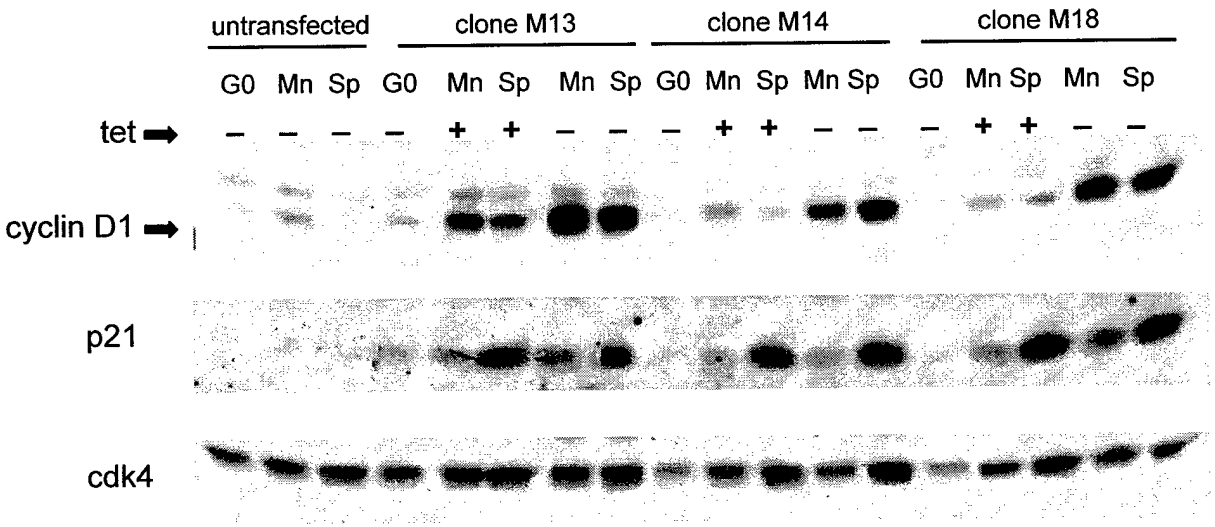
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Figure 1

Figure 2

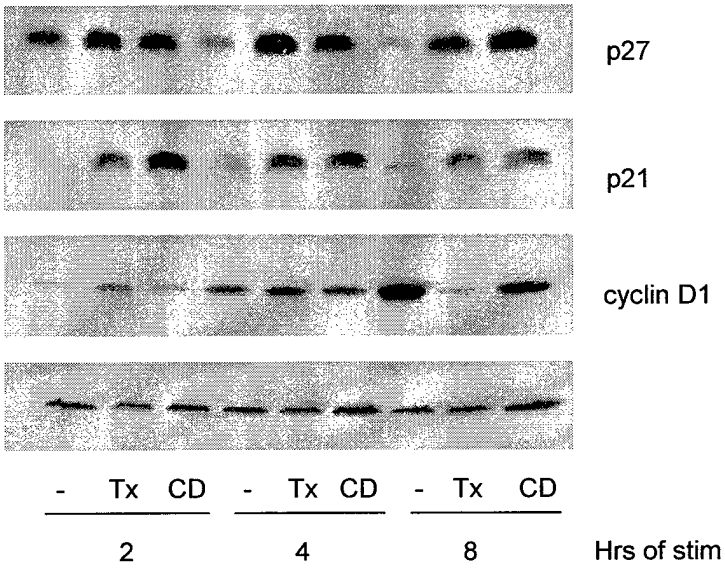
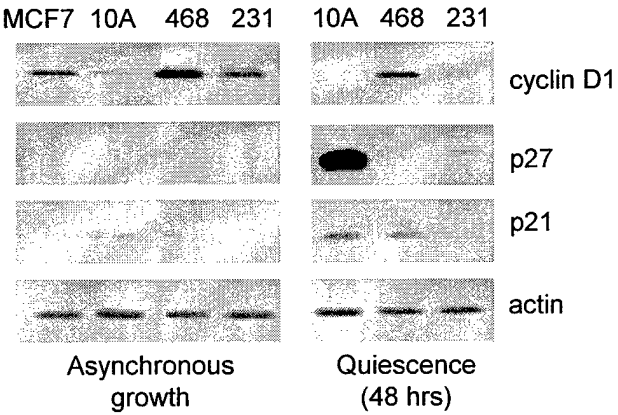


Figure 3



	High cyclin D1	Low p27	Low p21
Invasive	67%	45%	81%
In situ	25%	42%	100%

Table 1. Comparson of the expression levels of cyclin D1, p27kip1, and p21cip1 between invasive and in situ components in samples of human lobular carcinoma by immunohisotchemical stains. "high" cyclin D1, 2-3+; "low" p27/p21, 0-1+.

Invasive vs In situ levels	cyclin D1	p27	p21
greater	70%	9%	17%
less	10%	18%	0%
equal	20%	73%	83%

Table 2. Relative expression levels of cyclin D1, p27kip1, and p21cip1 between the invasive and corresonding in situ components of samples of human lobular carcinoma determined by immunohistochemical stains.

	High cyclin D1	Low cyclin D1	High p27	Low p27
High Ki-67	22%	29%	10%	75%
Low Ki-67	0%	14%	90%	25%

Table 3. Comparison between high versus low expression of cyclin D1 and p27 and the proliferation antigen Ki-67 in invasive components of human lobular carcinoma determined by immunohistochemical staining. "High", 3+; "Low", 0-1+.

Case	ER	PR	cyclin D1	p27
3	12	43	1+	2+
4	24	49	2+	2+
5	71	12	TI	3+
6	14	179	0	0
8	63	53	3+	1+
9	174	52	1+	1+
10	16	195	3+	1+
11	102	192	3+	1+
12	11	16	3+	3+
13	20	29	3+	3+

Table 4. Comparison of quantitative levels of estrogen and progesterone levels and the staining intensities of cyclin D1 and p27 by immunohistochemistry in cases of invasive lobular breast carcinoma. (TI, technically inadequate)

CYCLIN D1 AND P27 IN HUMAN LOBULAR CARCINOMA

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Derangements in the cell cycle machinery contribute to uncontrolled cell growth and tumorigenesis. Regulators of G1 progression including cyclin D1 and the cyclin-dependent kinase (cdk) inhibitor, $p27^{kip1}$, appear to be of particular importance in the pathogenesis of breast cancer. Overexpression of cyclin D1 in samples of human breast cancer is associated with a poor prognosis, but deliberate overexpression of cyclin D1 in breast cancer cell lines fails to reproducibly stimulate proliferation. Moreover, cyclin D1 is similarly expressed in both invasive and in situ components of human ductal carcinoma. These data suggest that cyclin D1 participates in, but is not sufficient to induce, breast tumorigenesis.

Relatively little is known about the pathogenesis of invasive lobular carcinoma (ILC), a histopathological entity distinct from invasive ductal carcinoma, comprising ~10% of breast cancer cases. To analyze the relationship between cyclin D1 and cdk inhibitors in vivo, we have analyzed 22 confirmed cases of human ILC samples immunohistochemically for cyclin D1 and $p27^{kip1}$ protein expression. When present in the specimens, components of lobular carcinoma in situ were also analyzed. Antibody staining was optimized using positive and negative controls derived from cell lines that under- or overexpress the corresponding protein. Samples were scored as 1+ (1-33% cells positive), 2+ (34-66%), or 3+ (67-100%). The results indicate that ~60% of ILC and 25% of in situ carcinoma samples overexpress cyclin D1 (2-3+). Cyclin D1 levels in the invasive component were greater than those in the corresponding in situ component in ~63% of cases. However, cyclin D1 levels did not correlate with levels of MIB-1, a proliferation marker, in either the invasive or in situ components. In contrast, $p27^{kip1}$ was expressed (2-3+) in 50% of the samples and there was a perfect correlation (100%) between the levels of $p27^{kip1}$ in the invasive and corresponding in situ components. There was also a strong correlation (90%) between the highest $p27^{kip1}$ expressers (3+) and low MIB-1, which increased to 100% when combined with low expressers (0-1+) of cyclin D1. In conclusion, the poor prognosis associated with overexpression of cyclin D1 does not appear to reflect effects on proliferation in ILC, whereas $p27^{kip1}$ expression appears to correlate inversely with proliferation. Unlike ductal carcinoma, cyclin D1 is preferentially overexpressed in invasive components of ILC.

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